Lipase-Catalyzed Solid Phase Synthesis of Sugar Esters*

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Lipase-catalyzed synthesis of sugar fatty acid esters was performed in a heterogeneous reaction system in the presence of an organic solvent serving as *adjuvant*. Although the sugar is almost insoluble in such a system, high conversions to the corresponding sugar esters were achieved, due to crystallization of the product. Acylation occurred regioselectively at the primary hydroxyl group and subsequent diacylation was observed only in the case of caprylic acid (2-5%). Best conditions were found for solvents having low log P values and low product solubility such as acetone, using immobilized lipase from *Candida antarctica* (CAL-B, Novo SP435) and fatty acids with chain lengths from C_{12} to C_{18} as acyl donors. The esterification of β-D(+)-glucose with stearic acid resulted in up to 100% conversion after 48 hours equal to a productivity of 0.4 mmol sugar ester per gram lipase and hour.

Lipase-katalysierte Synthese von Zuckerestern. Die Lipase-katalysierte Synthese von Zucker-Fettsäureestern erfolgte in einem heterogenen Reaktionssystem in Gegenwart eines organischen Lösungsmittels als *Adjuvant*. Obwohl die Zucker in diesem System nahezu unlöslich sind, wurden hohe Umsetzungen zu den entsprechenden Zuckerestern erzielt, was auf die Kristallisation des Produktes zurückzuführen ist. Die Veresterung erfolgte regioselektiv an der primären Hydroxylgruppe des Zuckers, eine nachfolgende Acylierung zum Diester wurde nur bei Caprylsäure (2-5%) beobachtet. Beste Reaktionsbedingungen ergaben sich bei Verwendung von Lösungsmitteln mit niedrigen log P-Werten, die eine geringe Produktlöslichkeit aufweisen (z.B. Aceton), mit Lipase aus *Candida antarctica* (CAL-B, Novo SP435) und Fettsäuren mit Kettenlängen zwischen C_{12} und C_{18} als Acyldonoren. Die Veresterung von β-D(+)-Glucose mit Stearinsäure ergab bis zu 100% Umsatz nach 48 Stunden, entsprechend einer Produktivität von 0,4 mmol Zuckerester pro Gramm Lipase und Stunde.

1 Introduction

Carbohydrate fatty acid esters are nonionic surfactants and are widely used in the cosmetic and food industry (e.g. low-fat spreads, sauces, ice-creams, mayonnaises etc.), due to their amphiphilic character, biodegradability and good surface properties. The (trans)esterification of unprotected sugar moieties with fatty acids is a high-temperature reaction and is performed in the presence of an alkaline catalyst [1], leading to a mixture of isomers of mono- and polyacylated sugar esters. A typical commercial product is sorbitol monostearate (Span™). Beside various other groups of biosurfactants, sugar fatty acid esters have also been produced by means of fermentation. These whole cell processes have the advantage of low production costs and great diversity, however, they suffer from a lack of chemo- or regioselectivity and high costs for purification [2-4].

In recent years, the use of isolated enzymes as a further alternative for the synthesis of sugar esters has been explored, allowing the regioselective synthesis of defined products. In these investigations monoacylated sugars have been the main target product [5].

A major difficulty here is the solubilization of sugars in organic media and a range of methods has been developed to overcome this problem. Only a few solvents are capable of solubilizing sugars (e.g. pyridine, 2-pyrrolidone, dimethylformamide). However, they cannot be used for food application. Several articles are dealing with the enzymatic formation of sugar esters in these organic solvents using activated acyl donors [6-7] or two-phase systems [8]. Another approach is based on the "hydrophobisation" of sugars by forming isopropylidene [9] or phenylboronic acid [10-11] complexes or by using alkylglucosides [12-14]. However, these methods require additional reaction steps increasing the overall costs.

Recently, it was shown that enzymatic reactions can be carried out in mainly solid reaction systems, e. g. for the synthesis of peptides by protease [15] and monoacylglycerides by lipase [16].

In this paper we describe the application of this method to lipase-catalyzed synthesis of sugar fatty acid esters in a mainly solid-phase system consisting of insoluble sugar, fatty acid and product. Main emphasis is put on the role of the organic solvent (*adjuvant*) maintaining a catalytic phase for the action of the enzyme.

2 Materials and Methods

2.1 Lipase and chemicals

Immobilized lipase from *Candida antarctica* (CAL-B, SP435, Novo, Bagsvaerd, Denmark, 50 U/g) was directly used without any pretreatment. α- D(+)-glucose, β-D(+)-gluclose, sorbitol, mannitol, lauric acid and myristic acid were obtained from Sigma, Deisenhofen, Germany. t-Butanol, dioxane, γ-butyrolactone, methanol, dichloromethane, ethylacetate, acetic acid, sulphuric acid, D(+)-galactose, D(+)-mannose and 3-methyl-3-pentanol were purchased from Fluka, Buchs, Switzerland. Acetone, acetonitrile and tetrahydrofuran were puchased from Riedel de Haen, Seelze, Germany. Palmitic acid was obtained from Merck, Darmstadt, Germany. Caprylic acid and caproic acid were gifts from Henkel, Düsseldorf, Germany.

2.2 Determination of the solubility of sugars and their esters in organic solvents

The compounds were added to 1 ml of organic solvent in a capped glass flask until saturated solutions were obtained. After incubation at 25°C or 60°C for 24 hours and centrifugation, the concentration of each compound was analyzed from the supernatant by HPLC (see below).

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2.3 General procedure for enzymatic reactions

The reaction mixture consisted of equimolar (typically 0.5 mmol) amounts of free carbohydrate and fatty acid and an organic solvent as adjuvant (in a concentration of 100-300% w/w). 20% (w/w of substrates) of an activated molecular sieve (4 Å, 10 mesh) were added in order to absorb the water generated during esterification. The reaction mixture was incubated in a capped vial, placed in an oil bath at 60°C and stirred by a magnetic bar (250rpm). The reaction was started with the addition of lipase. Samples from the suspension were taken at intervals, centrifuged and the supernatants were analyzed by thin-layer chromatography (TLC) and HPLC (see below). At the end of the reaction the mixture was extracted with two organic solvents (1st tetrahydrofuran, 2nd hexane), filtered and excess solvent was evaporated under vacuum. The monoacylated sugar ester thus obtained in a purity of 95% was further purified by silica gel column chromatography (solvent: ethylacetate and ethylacetate/methanol = 15/1 and 9/1 (v/v)). No considerable loss in yields occurred during purification; thus the conversions given in Tab. 2. and Tab. 3 resemble isolated yields. Position and degree of acylation were finally confirmed by NMR spectroscopy (data not shown). The purified sugar fatty acid esters were also used as standards for HPLC.

Productivities were calculated from the conversion and expressed as mmol product formed per gram enzyme per hour (mmol/g×h).

2.4 TLC Analysis

Thin-layer chromatography (TLC) analysis was performed on unreactivated silica gel plates $60F_{240}$(Merck) using chloroform:methanol:acetic acid:water = 70:20:8:2 (v/v/v/v) as developing system. The plates were sprayed with 50% sulphuric acid and heated at 110°C on a heating block for 5min to develop the sugar and sugar ester spots.

2.5 HPLC Analysis

All solutions and reaction mixtures were analyzed by a HPLC system (Sykam, Gilching, Germany) equipped with a Nucleosil 120-5C_{18}column. For the analysis of 6-O-β-D-glucose palmitate a mobile phase consisting of methanol:acetonitrile:water = 70:25:5 (v/v/v) was used; monoester and fatty acids were detected with a refractive index detector. For the analysis of all other carbohydrates, the mobile phase consisted of the following solvent system and gradient: A: methanol:water:acetic acid = 49.5:50:0.5 (v/v/v) and B: methanol. Analysis was performed at a flow rate of 1.0 ml/min at 39°C with a light scattering detector at 2.5 bar nitrogen pressure. For reasons of a low solubility of the product and a low solidification of the reaction mixture, the reaction course was determined by the decrease of fatty acid concentration in the liquid phase. Samples were taken directly from the reaction mixture and were treated as described before. The conversion thus determined was confirmed after workup of the reaction mixture from the isolated yields.

3 Results and Discussion

3.1 Selection of suitable organic solvents

A suitable organic solvent should dissolve enough substrate to allow an enzymatic reaction, but at the same time it should not affect the enzyme activity or stability. Moreover, the product solubility should also be low to favour an irreversible reaction by facilitating crystallization.

Initially, we have determined the solubility of glucose and 6-O-β-D(+)-glucose palmitate in various organic solvents at 25°C (Tab. 1). It turned out that the solubility of glucose never exceeded more than 0.44 mg/ml (for dioxane); the generated ester was more soluble (up to 8.1 mg/ml for tetrahydrofuran), but still large amounts of solvent would be necessary for a total solubilization of the reactants. Therefore, the term *adjuvant* [17] should be rather used, instead of organic solvent.

Tab. 1. Solubitity of β-D(+)-glucose and 6-O-β-D(+)-glucose palmitate in organic solvents.

Solvent	Solubility [mg/ml]* Glucose	Glucose palmitate
dioxane	0.44	2.2
acetonitrile	n.d.	<0.03
acetone	0.04	0.200
γ-butyrolactone	0.15	<0.03
tetrahydrofuran	0.02	8.1
dichloromethane	<0.002	n.d.
t-butanol	0.34	1.1
t-amylalcohol	0.39	1.6
3-methyl-3-pentanol	n.d.	n.d.

*: at 25°C

Surprisingly, by mixing solid sugar particles and fatty acid in the presence of small amounts of these adjuvants, high conversion to the corresponding sugar ester was achieved, e.g. 85% conversion in the formation of 6-O-β-D(+)-glucose palmitate catalyzed by lipase from *Candida antarctica* B (Novo SP435). This enzyme was found most suitable in a screening of several commercially available lipases (data not shown), although the specific activity of this immobilized lipase is rather low (50 U/g). Thus, in terms of Units only small amounts of lipase are necessary to achieve high conversions. At the early stage of the reaction (usually up to 4 hours), the product is accumulated in the liquid phase until saturation is achieved. Above this point, fine sugar ester particles crystallize from the reaction mixture and after approx. 24 h the mixture becomes solid. An equimolar mixture of sugar and fatty acid gave best results, an excess of fatty acid or sugar was found less suitable (data not shown).

Conversions achieved in all adjuvants are shown in Fig. 1 for the same reaction as a function of log P. The log P value is defined as the logarithm of the partition coefficient of a given solvent in an octanol-water mixture, and a correlation between enzymatic activity and solvent hydrophobicity has often been reported [18]. However, in our case no clear correlation between conversion and solvent hydrophobicity was observed. Adjuvants with log P <1.0 such as acetone, acetonitrile, dioxane and t-butanol seem to be most suitable. Next, reactions were performed at different adjuvant concentrations, as shown in Fig. 2 for acetone, tetrahydrofurane and 3-methyl-3-pentanol. In all cases, an optimum adjuvant concentration between 100–150% (w/w of total substrates) was found. Further increase of the adjuvant concentration led to a decrease in conversion. We assume that this is due to the increased solubility of the sugar ester produced, suppressing crystallization of the product.

Under optimized conditions up to 100% conversion was achieved in the synthesis of 6-O-β-D(+)-glucose palmitate. During the time course of the reaction, most product accumulates in the solid phase, while the free fatty acid disappears from the liquid phase (Fig. 3).

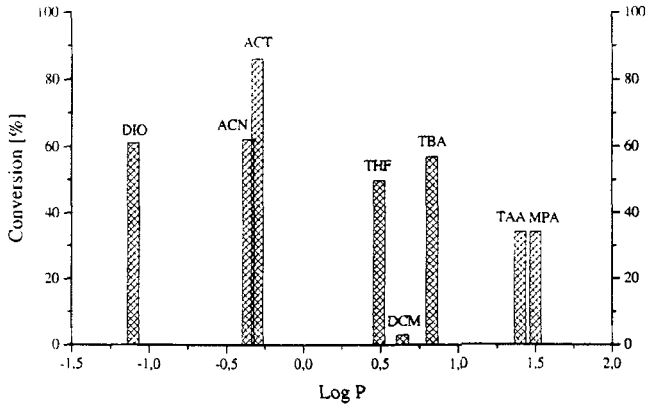

Fig. 1. Relationship between conversion and log P of various adjuvants. Reaction conditions: 0.5 mmol palmitic acid, 0.5 mmol β-D(+)-glucose, 20 mg CAL-B, 100 mg molecular sieve, 60°C, 200 rpm and 0.8 ml adjuvant, 96 h reaction time;
DIO-dioxane, ACN-acetonitrile; ACT-acetone, THF-tetrahydrofuran; DCM-dichloromethane; TBA-t-butanol; TAA-t-amylalcohol; MPA-3-methyl-3-pentanol.

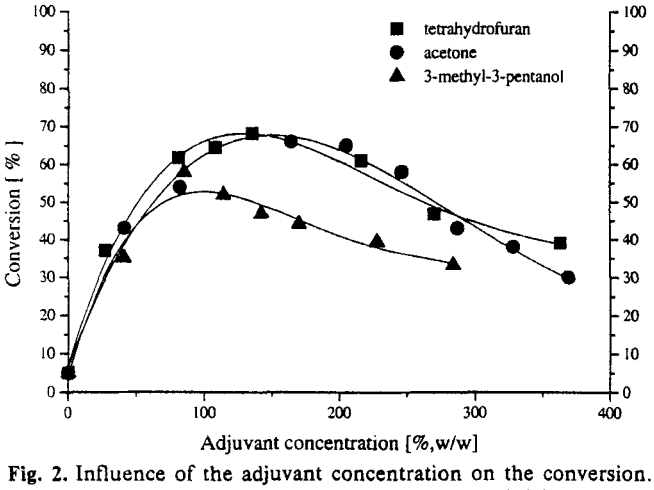

Fig. 2. Influence of the adjuvant concentration on the conversion. Reaction conditions: 1mmol palmitic acid, 1 mmol β-D(+)-glucose, 40 mg CAL-B, 200 mg molecular sieve, 60°C, 200 rpm and 0-1500 μl adjuvant.

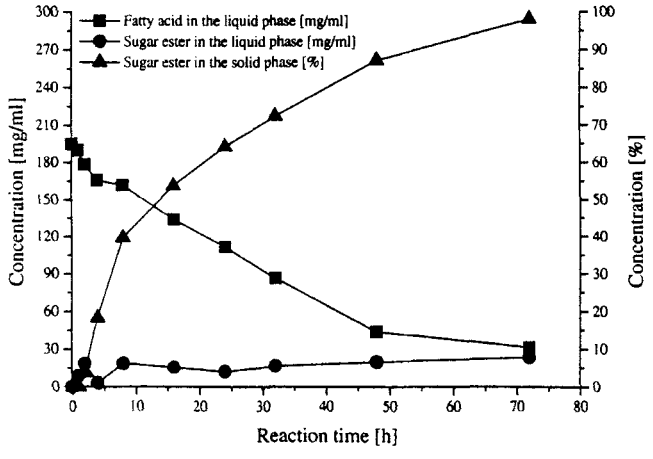
Fatty acid in the liquid phase [mg/ml]
Sugar ester in the liquid phase [mg/ml]
Sugar ester in the solid phase [%]
Concentration [mg/ml]
Concentration [%]
Reaction time [h]

Fig. 3. Time course of the synthesis of 6-O-β-D(+)-glucose palmitate. Reaction conditions: 5 mmol palmitic acid, 5 mmol β-D(+)-glucose, 200 mg CAL-B, 1.0 g molecular sieve, 60°C, 200 rpm and 5 ml t-butanol.

3.2 Use of other fatty acids as acyl donors

Several fatty acids ranging from C_8 to C_{18} were used as substrates and an inverse correlation between sugar ester solubility (at 60°C) and conversion was found (Tab. 2). The highest conversion was achieved for stearic acid (100%). With decreasing chain length a decrease in the conversion could be observed, especially for medium to short chain fatty acids like caproic (56%) and caprylic acid (50%). With caprylic acid no solidification of the reaction mixture occurred, either.

3.3 Use of other sugars as acyl acceptors

The lipase-catalyzed solid-phase synthesis of sugar esters was not restricted to β-D(+)-glucose. Several other unprotected monosaccharides were also acylated. However, the reactivity was quite different (Tab. 3). With α-D(+)-glucose and D(+)-mannose conversions between 62 and 69% were achieved after 72h of reaction time, but D(+)-galactose was a poor substrate and only 13% conversion was found (Tab. 3). It seems that the position of other hydroxy groups especially at position C4 influenced the lipase activity towards the primary hydroxy group at C6. Additionally, the hydroxy group at C1 also affects the lipase activity as found in the acylation of α- and β-D(+)-glucose.

Tab. 2. Conversions achieved in the acylation of β-D(+)-glucose using different acyl donors and solubility* of sugar ester produced in t-butanol or acetone.

Fatty acid	Solubility of sugar ester [mg/ml]		Conversion [%]
stearic acid	10[a]	4[b]	100
palmitic acid	14[a]	5[b]	85
myristic acid	26[a]	13[b]	70
lauric acid	41[a]	20[b]	67
caproic acid	60[a]	48[b]	56
caprylic acid	160[a]	75[b]	50

*: at 60°C; [a] in t-butanol; [b] in acetone.
Reaction conditions:
1 mmol β-D(+)-glucose, 1 mmol fatty acid, 50 mg CAL-B, 200 mg molecular sieve, 1 ml t-butanol, 60°C, 200 rpm, reaction time: 48 hours.

Tab. 3. Conversion achieved in the acylation of different sugars with palmitic acid.

Sugar	Conversion [%]
α-D(+)-glucose	69
β-D(+)-glucose	87
D(+)-mannose	62
D(+)-galactose	13
mannitol	35
sorbitol	97

Reaction conditions: 0.5 mmol palmitic acid, 0.5 mmol sugar, 20 mg CAL-B, 100 mg molecular sieve, 60°C, 200 rpm and 600 μl γ-butyrolactone; reaction time: 72 hours.

4 Conclusion

It was shown here, that some drawbacks like low solubility, reaction rate and productivity associated with the enzymatic biotransformation of polar sugars in dilute organic solvents can be easily overcome by performing those reactions in a system with solid substrates and products.

The correct choice and concentration of the organic solvent serving as adjuvant is likely to be most important in order to obtain high yields of sugar fatty acid esters.

A clear advantage of this reaction system is the use of readily available starting materials; no activated acyl donors or modified sugars are necessary in order to achieve high conversions in acceptable reaction times.

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Analytik und Biophysik von verzweigtkettigen Fettsäuren in Lipiden*

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Gegenstand der Untersuchungen sind langkettige Fettsäuren mit einer Methylgruppe in unterschiedlicher Position zur Carboxylgruppe sowie Fettsäuren mit unterschiedlich langen Alkylresten in α-Position. Die analytische Razemattrennung als Methylester mittels GC an einer chiralen Säule hängt stark von der Struktur der verzweigten Fettsäure ab. Präparativ wurden razemische α-verzweigte Fettsäuren als diastereomere Amide getrennt. Die verzweigten Fettsäuren wurden in 1,2-Diacyl-, 1-Acyl-2-hexadecyl- und 2-Acyl-1-hexadecyl-glycerophosphocholine eingebaut, deren physikochemisches Verhalten mit verschiedenen Methoden (Mikrokalorimetrie, Röntgendiffraktion) untersucht wurde. Durch HPLC und präparative Säulenchromatographie gelang die Trennung von diastereomeren Phospholipiden mit verzweigten Fettsäuren.

Analytics and biophysics of branched fatty acids in lipids. Objects of our investigations are long chain fatty acids with methyl groups in different positions and fatty acids with a different length of the side-group in α-position. The separation of racemic branched fatty acid methyl esters by gas chromatography on a chiral column depends on the structure of the branched fatty acid. Diastereomeric amides and phosphatidylcholines with α-branched fatty acid residues were separated preparatively. The branched fatty acids were incorporated into 1,2-diacyl-, 1-acyl-2-hexadecyl- and 2-acyl-1-hexadecyl-glycerophosphocholines and characterized by differential scanning calorimetry and X-ray diffraction.

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